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Phytochemical study, microbiological and cytotoxicity activity in *Artemia salina* Leach, aerial parts of *Petiveria alliacea* L. Phytolaccaceae.

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ABSTRACT: This study aimed to phytochemical study of the aerial parts of *Petiveria alliacea* L. (young branches and leaves), cytotoxicity evaluation (LC_{50}) of leaves in relation to *Artemia salina* L. and antimicrobial activity, which were used strains of gram-positive bacterium: *Staphylococcus aureus* and gram-negative: *Klebsiella pneumoniae* and *Escherichia coli*, these submitted to the methanol crude extract (MCE) of leaves, at the following concentrations: 25.50 and 100mg/mL. It was possible to identify in the young branches of *P. alliacea* L. the presence of organic acids, phenols and tannins in the alkaloids leaves, steroids and triterpenoids, saponins, phenols and tannins, where the activities of these metabolites match with some information alleged by the population. The leaves' Methanol Crude Extract showed LC_{50}=1709.77µg/mL, being nontoxic at the tested concentrations, whereas for plant extract in relation to *A.salina* are considered nontoxic when LC_{50}>1000µg/mL. The antimicrobial activity of the Methanol Crude Extract of the leaves showed inhibition only for the bacteria *Escherichia coli* at 100mg/mL concentration, and this activity may be related to the presence of phenols and tannins in the extract. The obtained results turn the species promising in search of secondary metabolites, but there is the need of further studies to identify its main active ingredients.

Keywords: Mucuracaa, Phytochemical Screening, Class of Compounds.

RESUMO: Estudo fitoquímico, atividade microbiológica e citotóxica em *Artemia salina* Leach. Das partes aéreas de *Petiveria alliacea* L. Phytolaccaceae. Este trabalho teve como objetivo o estudo fitoquímico das partes aéreas de *Petiveria alliacea* L. (galhos jovens e folhas), avaliação da citotoxicidade (CL_{50}) das folhas realizada frente à *Artemia salina* L. e atividade antimicrobiana, na qual foram utilizadas cepas de bactérias gram-positiva: *Staphylococcus aureus* e gram-negativas: *Klebsiella pneumoniae* e *Escherichia coli*, e estas submetidas ao extrato bruto metanólico (EBM) das folhas, nas seguintes concentrações: 25, 50 e 100 mg/mL. Foi possível identificar nos galhos jovens de *P. alliacea* L. a presença de ácidos orgânicos, fenóis e taninos, e nas folhas alcalóides, esteróides e triterpenóides, saponinas, fenóis e taninos, onde as atividades destes metabólitos condizem com algumas alegadas pela população. O Extrato Bruto Metanólico das folhas apresentaram CL_{50}=1709,77µg/mL, sendo então atóxica nas concentrações testadas, visto que para extrato de plantas frente a *A. salina*são considerados atóxicos quando CL_{50}>1000µg/mL. A atividade antimicrobiana o Extrato Bruto Metanólico das folhas apresentou inibição apenas para bactéria *Escherichia coli* na concentração de 100mg/mL, e esta atividade pode estar relacionada com a presença de fenóis e taninos no extrato. Os resultados obtidos tornam a espécie promissora em busca de metabólitos secundários, mas devem ser feitos estudos mais aprofundados para identificação dos principais princípios ativos da mesma.

Palavras-chave: Mucuracaa, Screening fitoquímico, Classe de compostos.

1. Introduction

Brazil is the country that holds the largest share of biodiversity of medicinal plants in the world, besides a considerable traditional knowledge about them, which is passed from generation to generation (LEÃO et al., 2007 apud PAULA; CRUZ-SILVA, 2010).

The use of medicinal plants in health recovery has evolved over time, from simpler forms of treatment, probably used by the

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caveman, to technologically sophisticated forms of industrial manufacturing used by modern man (LORENZI; MATOS, 2008).

Efforts and resources have been used in recent years to research the feasibility of research on composition and applicability of vegetal species, traditionally used to treat different symptoms (BARBOSA, 2011).

The constituency of Phytolaccaceae is controversial; the number of genera and species varies according to different classification systems (ENGLERT&PRANTL, 1894; CRONQUIST, 1981; JUDD et al., 2002; APG II, 2003). Cronquist (1981) considered the family with 18 genera and 125 species and included the genus *Microtea* in Chenopodiaceae. According to Judd et al*.,* (2002), Phytolaccaceae consists of four genera and 30 species, the largest genus is *Phytolacca* (23 species).

The Phytolaccaceae family is pantropical and occurs, mainly, in South America. In Brazil, it is represented by nine genera (BARROSO, 1978), five of them occur in Rio Grande do Sul (*Phytolacca, Microtea, Petiveria, Rivina* and *Seguieria).* They are herbaceous-subshrub plants, rarely arboreal, preferably associated with forest environments. According to Judd et al*.,* (2002) the representatives of Phytolaccaceae are typically entomophilous (their flowers attract bees, wasps, flies and butterflies) and their fruits are dispersed by birds (NEVES et al., 2006).

The vegetal species *Petiveria alliacea* L. from the family Phytolacaceae, is a native plant from the Amazon, known primarily as opossum herb (CAMARGO, 2007). It is an erect herbaceous plant, perennial, rhizomatous, with slight aroma of garlic, of about 70 cm, with discrete flowers, is widely grown in home gardens (LORENZI; MATOS, 2008).

Subshrub, woody base, erect, with long branches, delicate and upward; short-petiolate leaves, alternate, stipulated, membranous, acute at the apex, narrow at the base, small sessile flowers, clustered in axillary inflorescences and spiciformis ending; androecium with four stamens, unicarpellate gynoecium with superior ovary; achene cylindrical fruit, flat and crenulate (CAMARGO, 2007).

The leaves of *P. alliacea* have from 5 to 10 cm long and 2 to 6 cm wide, are discolor, oblong-lanceolate, acuminate, integerrimus, wedge base and short petioles. The texture of the leaves varies from membranous to herbaceous. The main midrib is prominent on the abaxial, while the secondary midrib is arched. The stem is cylindrical; the majority of the body is an herbaceous constitution with woody base only (ROCHA et al., 2006).

This species has been widely used in various applications such as, for example, antirheumatic, antineoplastic, anti-influenza, antitussive, analgesic, insecticide ascaricide, bactericide and fungicide (GUEDES, 2009).

The chemical constitution, in addition to the essential oil, has coumarins, saponins, petiverina, fatty acids, β-sitosterol, dibenzyl trisulphide, diallyl trisulphide, especially the organic sulfates, sodium nitrate, flavonoids, tannins and benzylthiol (OLIVEIRA, et al., 2010).

Artemia salina, a microcrustacean widely used as an indicator of toxicity of substances and crude extracts of plants using the Lethal Concentration (LC50) as a parameter for evaluating the biological activity of cytotoxicity, the Lethal Dose (LD50) as lethal activity and death curve for evaluation of doses and concentrations of natural products, avoiding the use of mice, as the sensitivity test results of these crustaceans are closer to reliably in human tests (DINIZ et al., 2012). Meyer et al., (1982) *apud* Araújo et al., (2010) considered significant (toxic) values less than 1000 mg/μL.

It is necessary to search for new antimicrobial agents, due to the appearance of resistant microorganisms and fatal opportunistic infections, associated with AIDS, chemotherapy and transplants. The study of antimicrobial agents has great coverage, important in various sectors of the pharmaceutical and cosmetic filed. Another point to be emphasized is the use of this study as the first screening in the discovery of the

pharmacological activity of new agents, it is extremely important, especially in a country like Brazil that offers immense biodiversity. Thus, research on antimicrobial agents may contribute to the development of health worldwide, by finding substances more effective and less toxic against the resistance and the appearance of pathogenic microorganisms (OSTROSKY, 2008).

Therefore, this study aimed to the phytochemical study of the aerial parts of *Petiveria alliacea*L. (young branches and leaves), cytotoxicity evaluation (LC_{50}) and antimicrobial activity of the leaves of this plant.

2. Material and methods

Vegetal Material

The vegetal species (*Petiveria alliacea* L.) was collected in Pedra Branca do Amaparí - AP. The identification of vegetal material was made by Dr. Wegliane Campelo da Silva Aparício, at the Herbarium of Federal University of Amapá (HUFAP).

Preparation, drying of the vegetal material and obtaining the methanol extract

The aerial parts (young branches and leaves) of the vegetal material were dried in a stove for two days at temperature of, approximately, 45°C, to eliminate water and inhibition of proliferation of microorganisms. After dried, it was milled in a grind mortar with the help of pistil, obtaining young branches powder (14.49 g) and leaves (35.96 g) to decrease the particles and to increase the surface area of contact, facilitating the extraction, later stage. After dried and milled, the aerial parts were submitted, separately, to the hot extraction under reflux, using methanol as solvent (500 mL and 750 mL, for young branches and leaves, respectively) followed by filtration and concentration in evaporator route, to removing the solvent and obtaining the methanol crude extract of young branches (2.2 g) and leaves (6.3 g).

Preliminary phytochemical analysis

Phytochemical analysis was performed on extracts of young branches and leaves of *Petiveria alliacea* L., aiming at the identification of secondary metabolites. With MCE of young branches, there were performed the following tests for: flavonoids, organic acids, phenols/tannins, steroids/triterpenoids, depsides/depsidones; and for MCE of leaves besides these (except depsides/depsidones) it was also conducted to: alkaloids and reducing sugars, polysaccharides and saponins, according to the methodology described by Matos, 1997 *apud* Silva, Miranda and Conceição (2010).

Toxicity in Artemia Salina Leach

The cytotoxic activity of the extracts was evaluated by testing lethality of *Artemia salina* Leach, according to the method proposed by Meyer et al., 1982 apud Nascimento et al., 2008. Eggs of *Artemia salina* were placed in artificial sea water at room temperature for 48 hours. A solution was prepared with the MCE of leaves of *P alliacea* L., and this solution was placed in tubes in six different concentrations, the assay was done in triplicate. After 48 hours, the hatched larvae were placed in the tubes containing the extract solution and in the control test tubes that had only artificial sea water and Tween 80. The counting of the number of dead larvae was measured after 24 hours and this number was used for calculation of LC_{50} using the software Probit.

Antimicrobial analysis

For the microbiological test, the disk diffusion method was used according to the method Kirby-Bauer modified UT3, *apud* Estevam et al., 2009. It was used concentrations of 25mg/mL, 50mg/mL and 100mg/mL of the methanol crude extract of *Petiveria alliacea* L. for the following bacterium: *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC 25923).

The extract was diluted in methanol at concentrations of 25, 50 and 100 mg/mL, immediately after, it was impregnated in sterile filter paper discs. After evaporation of methanol, the filter paper discs were placed in

petri dishes containing the used bacteria, they were placed on a separated plate and around them there were antibiotics to control. The plates were kept in a bacteriological incubator at 37°C, for 24 hours, after this period there were made the measurement of halo inhibition of bacterial growth, and, thus, it was determined whether or not a there was a possible antimicrobial activity.

3. Results

Through phytochemical screening of the methanol extracts of the aerial parts of the species *P. alliacea* L., it was possible to identify in young branches the presence of organic acids, phenols and tannins, and in the leaves it was identified alkaloids, steroids, triterpenoids, saponins, phenols and tannins (Table 1).

Table 1. Results of the phytochemical study of methanolic crude extracts of *Petiveria alliacea* L.

Class of secondary metabolites	Young branches	Leaves
Flavonoids	-	-
Organic Acids	+	-
Phenols and Tannins	+	+
Steroids and Triterpenes	-	+
Depsides and depsidones	-	0
Saponins	0	+
Reducing Sugars	0	-
Alkaloids	0	+
Polysaccharides	0	-
Coumarin derivatives	0	0
Anthraquinones	0	-
Purines	0	0
Resine	0	0
Carotenoids	0	0

Used parameters: (0) Not performed; (+) Present; (-) Absent

The MCE of leaves of *P. alliacea* L. presented LC_{50} 1709.77µg/mL, nontoxic at tested concentrations, and in relation to antimicrobial activity the MCE of leaves had inhibition only for the bacteria *Escherichia coli* at concentration of 100mg/mL.

4. Discussion

The activities of secondary metabolites are consistent with some information alleged by the population, and they present the following actions: Organic acids have antifungal action, phenols have antibacterial and antiviral, tannins are antifungal, antifungal and antirheumatic, alkaloids have antitumor and antitussive action (SIMÕES, 2010), steroids and triterpenoids have analgesic activity (SILVA, 2005 *apud*RODRIGUES, et al., 2010) and saponins have antiviral action (SIMÕES, 2010).

Antimicrobial activity may be related to the presence of phenols and tannins in the extract. According to the obtained results, this study demonstrates that the species is promising in the search for chemical constituents with biological potential, and further studies are needed to obtain the main active principles.

Organic acids presentantifungal activity described in literature (SIMÕES, 2010), are largely used in food industry as additives, they can act like since antimicrobialuntil antioxidantagents (FIORUCCI; SOARES; CAVALHEIRO, 2002). These same were detected in methanolbrute extract of young branches, what justifies, in part, the use of the vegetal species for these activities.

Carboxylicacids have important organoleptic properties, so that characteristic sour taste was the first criteria for classification of these compounds. Acids with four to eight carbon atoms has unpleasant smell (FIORUCCI; SOARES; CAVALHEIRO, 2002), sotheir presence can be responsible, jointly to organic sulfides, for the characteristic scent of *P. alliacea* species.

Phenols and tannins presents proved activity as fungicide action, because *in vitro* tests realized with extracts rich in tannins or with pure tannins have identified potent fungicide action. It's believed that their activities are due to, by part, the ability to form complexes with other molecules including macromolecules, as proteins and polysaccharides (SIMÕES, 2010), in this way, by interacting with the membranes of

microorganisms, they can break up the biological barriers, causing the cellular content to extravasate and, consequently, its death. Thus the presence of these secondary metabolic reinforcesthe use of *P. alliacea* for these activities, that requires, however, performingof studies with their active principles isolated and purified. Remembering that the presence of organic acids were detected only in young branches, while phenols and tannins were found both in young branches and in leafs.

Alkaloids are considered an important class of secondary metabolic, because they are associated to a wide range of biological activities and for being considered toxic to humans by its chemical constitution. For example, in plants they act as natural protectors against attacks of decomposers agents, microorganisms and insects due to its bitter taste and its toxicity. In contact with cellular membrane, they interact direct with the lipidic double layer, probably by bridge of unsaturated fats phospholipids contained in the membrane, causing cellular death. Such antibacterial, antifungal, acaricidal and insecticidal activities can be associated with the report of use of species by traditional communities (MIURA, 2009; SIMÕES, 2010). On the other hand, some kinds of alkaloids act as specific agents (antitumor) of cellular cycle and block the cellular mitosis and inhibition of enzymes present in high concentrations in tumors (MESQUITA, 2009).

Steroids present many pharmaceutical actions, pointing out the direct effect in cardiac diseases reduction, due to its ability to lower serical levels of cholesterol. This class of metabolic is important, because it presents antibacterial, anti-inflammatory, antiulcerative and antitumor actions (LI; BEVERIDGE; DROVER, 2007).

The presence of triterpenes in the EBM of species come to awaken the study for use as insecticidal agent, once that further works can quantify and identify major components present in the extracts of branches and leafs of *P. alliacea* species (ANDRADE FILHO et al*.,* 2010).

Without mentioning that steroids and triterpenes have analgesical activity (SILVA, 2005 *apud* RODRIGUES, 2010) partially justifying the use of this vegetal species by traditional communities.

Saponins are important components for the action of many vegetal drugs, pointing out that traditionally used as expectorants and diuretics. However, these activities mechanisms are not completely elucidated. Some authors infer that respiratory tract irritations raise the volume of respiratory fluid, hydrating the bronchial secretions. The mucus would have, then, its viscosity lowered. Another possibility would be due to the superficial activity of saponinas, also generating lower viscosity and high ease of excretion of mucus. The diuretic activity, however, will be due to renal epithelium irritation caused by saponinas (SIMÕES, 2010). These mechanisms of action of saponins justify the utilization of the vegetal species *P. alliacea* as antigripal and antitusive agent by traditional communities, once in this work was possible to verify the presence of secondary metabolic.

Therefore, the detection of secondary metabolic classes in *P. allicea* species justify its traditional medical use, once that analysis for determination of its active principles are necessary, and a bio-monitored study for evaluation of biological activities and, mainly, of its toxicity.

An easy-executing bioassay, low cost and efficient in determining the acute toxicity of substancesis the essay about *Artemia salina* Leach, a micro-crustacean used in larval stage, very dependent of the environment where it is localized and, therefore, sensible to bioassays. The technique and its systematic utilization, as a form of obtaining active substances of vegetal extracts, is described by Meyer et al. Extracts with CL50 <1000 μg/mL are appropriated for bio-monitoring using this test, and extracts with CL50 >1000 μg/mL are considered non-toxic (NASCIMENTO, et al,. 2008). Substances whose CL50 are in the range of 80 μg/mL< CL50 > 250 μg/mL can present tripanomicid activity, in the other hand, substances with toxicity CL50 <145

µg/mL can present anti-tumor activity (GALOTTA; BOAVENTURA, 2005). The EBM of leafs of *P. alliacea* L. presented CL50 1709,77 µg/mL, being then non-toxic in the tested concentrations, although in the vegetal species have been detected classes of substances that presents some biological activity proved cited above, this non-toxicity can be explained, probably, by the low concentration of the substance present in the extract, not being possible, in mixture with many others, present toxic action, what would present with studies with fractions and isolated substances.

Concerning to antimicrobial activity, the EBM of leafs presented inhibition only the bacterium *Escherichia coli* in the concentration of 100 mg/mL, that is, in the higher concentration, and such activity can be related with the presence of phenols, tannins or alkaloids in the extract, once that for a better evaluation, an analysis of Minimal Inhibitory Concentration could present a better explaining, being therefore, the next stage of the project.

According to the obtained results, the present study shows that the species is promising in the search of chemical constituents with biological potential, requiring more profound studies for the obtaining of the main active principles and in the structural determination, as well as a bio-monitoring of the claimed activities by the traditional communities.

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6. References

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